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Research Paper

Morphological and molecular characterization of *Fusarium. solani* and *F. oxysporum* associated with crown disease of oil palm

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Abstract

Crown disease (CD) is infecting oil palm in the early stages of the crop development. Previous studies showed that *Fusarium* species were commonly associated with CD. However, the identity of the species has not been resolved. This study was carried out to identify and characterize through morphological approaches and to determine the genetic diversity of the *Fusarium* species. 51 isolates (39%) of *Fusarium solani* and 40 isolates (31%) of *Fusarium oxysporum* were recovered from oil palm with typical CD symptoms collected from nine states in Malaysia, together with samples from Padang and Medan, Indonesia. Based on morphological characteristics, isolates in both *Fusarium* species were classified into two distinct morphotypes; Morphotypes I and II. Molecular characterization based on IGS-RFLP analysis produced 27 haplotypes among the *F. solani* isolates and 33 haplotypes for *F. oxysporum* isolates, which indicated high levels of intraspecific variations. From UPGMA cluster analysis, the isolates in both *Fusarium* species were divided into two main clusters with the percentage of similarity from 87% to 100% for *F. solani*, and 89% to 100% for *F. oxysporum* isolates, which was in accordance with the Morphotypes I and II. The results of the present study indicated that *F. solani* and *F. oxysporum* associated with CD of oil palm in Malaysia and Indonesia were highly variable.

Key words: crown disease, *Fusarium solani, Fusarium oxysporum*, IGS-RFLP, morphological characteristic.

Introduction

Crown disease (CD) is a disease of oil palm that is prevalent in young palms between 1 to 3 years old and has been reported from oil palm plantations worldwide (Monge *et al.*, 1994; Corley and Tinker, 2003). The common symptoms of CD are rachis bending, breaking and rotting of some spears and growing leaves (Chinchilla, 2008). *Fusarium solani* and *F. oxysporum* were among the most frequent microorganisms isolated from the disease symptoms (Monge *et al.*, 1994).

F. solani is a cosmopolitan species and is classified into the section Martiella (Booth, 1971). *F. solani* can be distinguished into 50 subspecific lineages and most of them have not been further described formally (O'Donnell, 2000). The species is among a well known plant pathogen, causing various types of diseases on a wide range of plants and there are at least 111 plant species from 87 genera that are commonly infected by *F. solani* (Kolattukudy and Gamble, 1995).

F. oxysporum is a cosmopolitan species that are widely spread in all types of soil worldwide (Burgess, 1981). They are economically important species as they caused severe vascular wilts and root rot diseases in various crops (Nelson *et al.*, 1981) and have been reported as an emerging human pathogen, especially to immunocompromised individuals (Vartivarian *et al.*, 1993).

The identification of *Fusarium* species is mainly based on distinctive characters of the shapes and sizes of macro- and microconidia, presence and absence of chlamydospores as well as colony appearances, pigmentations and growth rates on agar media (Leslie and Summerell, 2006). Polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) of intergenic spacer (IGS) re-

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gion is commonly used as the IGS region appears to be rapidly evolving spacer regions (Hseu *et al.*, 1996). This technique is highly dependable for the differentiation of strains at the intraspecific level in *Fusarium* taxonomic studies (Hillis and Dixon, 1991; Mirete *et al.*, 2003).

The present study was conducted to identify and characterize *Fusarium* species by using morphological characteristics and to assess the genetic diversity of the *Fusarium* species recovered from oil palm plants showing CD symptoms in Malaysia and Indonesia.

Materials and Methods

Sampling and fungal isolation

Samplings of fronds and leaves of young (1 to 3 years old) oil palm plants showing CD symptoms (Figure 1) were conducted in several oil palm estates in Malaysia, namely Johor, Kedah, Kelantan, Perak, Negeri Sembilan, Melaka, Pahang, Selangor and Terengganu, and in Padang and Medan, Indonesia.

The fronds and leaves were surface sterilized by using 70% ethanol, cut into small pieces (2.0 x 2.0 x 2.0 cm), soaked into 1% sodium hypochlorite for 3 min, and rinsed for 1 min in several changes of distilled water. The surface-sterilised pieces were plated onto peptone pentachloronitrobenzene agar (PPA) (Nash and Synder, 1962), incubated at 25 ± 2 °C with 12 hours periods of fluorescent and black lights for 7 days (Salleh and Sulaiman, 1984).

Morphological identification and characterization

Species identification was based on the morphological characteristics of single-spored isolates as described by Booth (1971), Gerlach and Nirenberg (1982), Nelson *et al.* (1983), Burgess *et al.* (1994), and Leslie and Summerell (2006).

For microscopic characteristic, the isolates were cultured onto carnation leaf-pieces agar (CLA) for 2 to 4 weeks (Fisher *et al.*, 1982). Fifty macroconidia were observed randomly, and the width and length were measured. Soil agar (SA) was used to enhance the formation of the chlamydospores (Klotz *et al.*, 1988). Both CLA and SA were incubated under the incubation conditions described by Salleh and Sulaiman (1984) for at least 2 weeks.

For macroscopic observation, the cultural appearances (colony colour and pigmentations) were observed on potato dextrose agar (PDA). Colony colours and pigmentations were determined by using Methuen handbook of colour chart (Kornerup and Wancher, 1978).

Statistical analysis

The two - samples T - test was used to analyze the length and width of the macroconidia and the growth rates, by using MINITAB® statistical software version 15.

IGS-RFLP analysis

All isolates were grown in potato dextrose broth for 7 days at 25 ± 2 °C and approximately 100 mg mycelia were harvested and freeze-dried for 48 hours and ground into a fine powder using liquid nitrogen in a sterile mortar. The DNeasy® Plant Mini Kit (QIAGEN, Germany) was used to extract the DNA according to the manufacturers' instruction.

The IGS region was amplified by using CNL12 (5'-CTG-AAC-GCC-TCT-AAG-TCA-G-3') and CNS1 (5'-GAG-ACA-AGC-ATA-TGA-CTA-CTG-3') primers (Appel and Gordon, 1995). PCR amplification reactions were conducted in a 25 µL reaction mixture containing 5 µL 5 x buffer, 2.5 mM $MgCl_2$, 0.16 mM deoxynucleotide triphosphate (dNTPs) (Promega, USA), 0.7 µM each primer, 1.75 units of *Taq* DNA polymerase (Promega, USA),

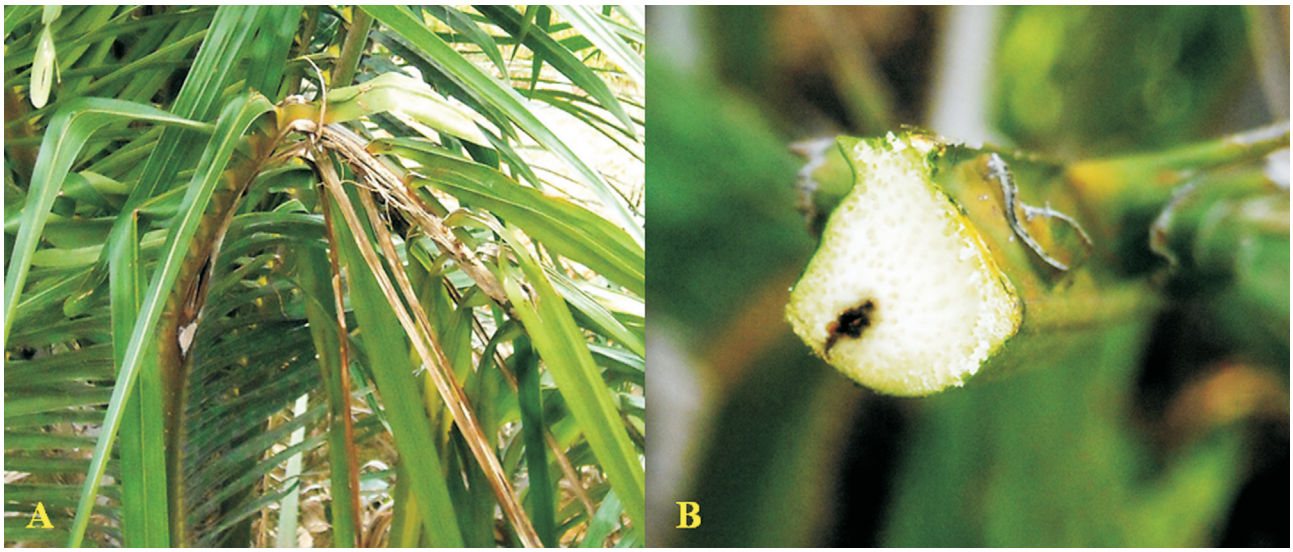

Figure 1 - CD symptoms on rotting leaflets (A) and cross section of rotting frond (B).

and 5 ng of template DNA. Each reaction was overlaid with 25 μL of sterilized mineral oil.

PCR was performed by using Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) with the following amplification cycles: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 55 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 2 min. Final extension was set at 72 °C for 7 min.

Seven restriction enzymes were used for restriction analysis, namely *Alu*I, *Bsu*RI, *Hin*6I, *Msp*I, *Pst*I, *Scr*FI and *Taq*I (Fermentas, USA). A total volume of 15 μL reactions were prepared, containing 1 x buffer, 1 U of restriction enzymes, and 7 μL of PCR products. The PCR products were digested with each of the restriction enzymes according to the manufacturers' instruction.

The restriction fragments were scored on the basis of presence (1) or absence (0) of a particular fragment. The binary data was then constructed to generate a similarity matrix based on Simple Matching Coefficient (SMC) (Romesburg, 1994) and unweighted pair group method with arithmetical mean (UPGMA) was used to perform the cluster analysis to determine the relationship among the isolates of both *F. solani* and *F. oxysporum*. Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) software package version 2.1 (Rohlf, 2000) was used to analyse the data and in the UPGMA cluster analysis, *F. proliferatum* was used as an outgroup.

IGS-RFLP products were detected on 1% agarose gel with Tris Borate EDTA (TBE) as a running buffer. Gel electrophoresis was conducted at 80 V, 400 mA, for 90 min and stained with ethidium bromide. The size of the amplified IGS fragment was estimated using 1 kb DNA ladder (GeneRulers, Fermentas, USA). Digested fragments were separated on 2% of agarose gel in TBE buffer with electrophoresis condition of 80 V, 400 mA, for 140 min and stained with ethidium bromide. The sizes of the restriction fragments were estimated using 100 bp DNA ladder (GeneRulers, Fermentas). The gels were visualized and photographed under UV transilluminator of SnapGene Photo Imaging System (SynGene).

Results and Discussion

From 350 samples of oil palm showing CD symptoms collected, a total of 131 isolates of *Fusarium* were recovered and identified into five species. Based on the morphological identification, the *Fusarium* species identified were *F. solani* (51 isolates), *F. oxysporum* (40 isolates), *F. semitectum* (21 isolates), *F. proliferatum* (12 isolates), and *F. subglutinans* (7 isolates). Based on the literatures, *F. solani* and *F. oxysporum* were the most frequently isolated and the most common species associated with CD symptoms. Thus, both most frequent *Fusarium* species *i.e. F. solani* and *F. oxysporum* were selected for further study.

Morphological characterization of *F. solani* isolates

Based on morphological characteristics, all the isolates can be divided into two different Morphotypes, I and II comprising 33 and 18 isolates, respectively (Table 1).

Morphotype I produced macroconidial septation from 3 to 5, whereas macroconidia septation in Morphotype II from 3 to 7. Shorter macroconidia was shown by Morphotype I with the length from 27.0 to 37.3 μm, while

Table 1 - Macroscopic- and microscopic characteristics of morphotypes I and II of *F. solani* isolates associated with CD of oil palm in Malaysia.

Morphological characterization	*F. solani*	
	Morphotype I	Morphotype II
Macroscopic characteristic		
Colony colour[a]	Pale to brown, brown-greenish to white-greenish	White-creamy to white-greyish
Pigmentation[b]	Pale brown yellowish brown with dark brown zonation	Colourless, white-creamy with dark brown zonation
Growth rate (cm)[c]	3.3 ± 0.2	3.5 ± 0.2
Microscopic characteristic		
Mean length of macroconidia (μm)[d]	34.4 ± 9.8	42.0 ± 3.0
Mean width of macroconidia (μm)[d]	3.7 ± 0.5	4.7 ± 0.5
Conidiogenous cell	Long and branched monophialides	Long monophialides
Production of chlamydospores	Sparsely produced on SA	Abundantly produced on CLA
Presence of sporodochia	Cream and blue	Cream
Macroconidia septation	3-5	3-7

[a]Colony colour were determined by observing the upper surface of the colony.
[b]Pigmentation were determined by observing the lower surface of the colony.
[c]Growth rates were taken after 3 days of incubation at 25 °C.
[d]Mean values of length and width of 50 randomly picked macroconidia ± standard deviation.

isolates in Morphotype II were longer *i.e.* from 36.6 to 46.2 μm. Narrow macroconidia were observed in isolates of Morphotype I; from 3.1 to 4.3 μm, while wider macroconidia (3.7 to 5.3 μm) in Morphotypes II. Both the length and width of macroconidia showed significant difference ($p < 0.05$).

Morphotype I produced sparse to abundant cottony mycelium with pale brown to brown, brown-greenish to white-greenish aerial mycelium, and the pigmentations were pale brown to yellowish brown with a dark brown zonation. On the other hand, Morphotype II produced sparse to abundant cottony mycelium with white-creamy to white-greyish colour of aerial mycelium and pigmentations from no pigments to white-creamy, with dark brown zonation. There was significant difference ($p < 0.05$) on the growth rate between isolates in Morphotypes I and II, in which the growth rate in Morphotype I was 3.3 ± 0.2 cm and Morphotype II was 3.5 ± 0.3 cm.

The feature of conidiogenous cell with branched and long monophialides were commonly observed in Morphotype I, while only a single long monophialides produced in Morphotype II. Morphotype I showed the ability to produce two colours of sporodochia, cream and blue, whereas only cream sporodochia was observed in Morphotype II. Chlamydospores in Morphotype I were sparse and can only be observed when cultured onto SA after 4 weeks, while isolates in Morphotype II produced abundant chlamydospores and can be easily seen on CLA after 2 weeks of incubation (Figure 2).

Some morphological characteristics that were described by Gerlach and Nirenberg (1982) and Nelson *et al.* (1983) were found in Morphotype II, but not in Morphotype I. Those characteristics were, the texture of the mycelium, colony colours and pigmentations, features of conidiogenous cells which are long monophialides, presence of cream sporodochia, and abundant of chlamydospores on CLA. Presence of chlamydospores was also used to group the isolates into Morphotypes I and II. So far, there has been no report on isolates of *F. solani* that slowly and sparsely produced chlamydospores as shown by isolates in Morphotype I. Absence of chlamydospores was reported by Zaccardelli *et al.* (2008) on *F. solani* isolates from various crops such as potato, chickpea, wheat, rice, melon, olive and soil.

IGS-RFLP analysis of *F. solani* isolates

A fragment size of 3000 bp was successfully amplified from all isolates of *F. solani*. The IGS fragment was digestible by seven restriction enzymes, except 15 isolates were not digested by using *Pst*I. Based on the restriction patterns, all the isolates were assigned into 27 haplotypes comprising one to seven isolates.

From UPGMA cluster analysis, all isolates of *F. solani* can be divided into two main clusters (A and B) with percentage of similarity from 87% to 100% (Figure 3). The two main clusters were in accordance to Morphotype I (33 isolates) in cluster A and Morphotype II (18 isolates) in cluster B. Percentage of similarity of the isolates in Morphotypes I and II were from 87% to 100% and from 89% to100%, respectively (Figure 4).

Twenty-seven haplotypes were assigned among the *F. solani* isolates, indicating that the isolates of *F. solani* associated with CD of oil palm in Malaysia are highly variable. Other study by using different molecular techniques has also shown that *F. solani* isolates are highly variable such as *F. solani* from banana, barley, soil and wheat in Ethiopia using AFLP (Bogale *et al.*, 2009) and *F. solani* from pine, tomato, onion, soil and air in Brazil by PCR-ribotyping and PCR-fingerprinting (Brasileiro *et al.*, 2004).

Morphological characterization of *F. oxysporum*

From the microscopic characteristics, all the isolates can be divided into two different morphotypes, I and II comprising 17 and 23 isolates, respectively (Table 2).

Differences were observed on the macroconidial shapes and sizes, the apical and basal cell morphology as well as cultural appearances. Macroconidia in Morphotype I was more common with straight and relatively slender in shape with a tapered and curved apical cell while the basal cell was pointed (Figure 4a). Majority of the isolates in Morphotype II have slightly curved and relatively thick macroconidia with a slightly hook apical cell and foot-shaped basal cell (Figure 4b). The macroconidial septation of Morphotype I ranged from 3 to 7, whereas macroconidia septation in Morphotype II ranged from 3 to 5. In both Morphotypes, 3-septate macroconidia were commonly observed. A significant difference ($p < 0.05$) was observed in length and width of the macroconidia in both Morphotypes. Longer macroconidia was shown by Morphotype I (35.7 to 48.8 μm) than those by Morphotype II (31.9 to 39.9 μm). Narrower macroconidia were observed in Morphotype I (3.2 to 4.7 μm) than those in Morphotypes II (3.9 to 4.9 μm) (Table 2; Figure 4).

The cultural appearances of Morphotype I isolates were sparse to abundant cottony mycelium with colony colour and pigmentations from pale violet, dark violet to peach-violet. Morphotype II produced sparse to abundant cottony mycelium with white to white-violet aerial mycelium and the pigmentations were from colourless to pale violet. There was no significant difference ($p < 0.05$) on the growth rate between isolates in Morphotypes I and II, 4.3 ± 0.7 and 4.5 ± 0.5 cm, respectively (Table 2). The separation of all 40 isolates into two distinct Morphotypes was based on the differences of macroconidial characters. The length and width of macroconidia in Morphotypes I and II were also significantly different ($p < 0.05$)

Gerlach and Nirenberg (1982) and Nelson *et al.* (1983) described the colony appearances on PDA of *F. oxysporum* as highly variable. The peach-violet colony colour and pigmentations observed in isolates of Morphotype

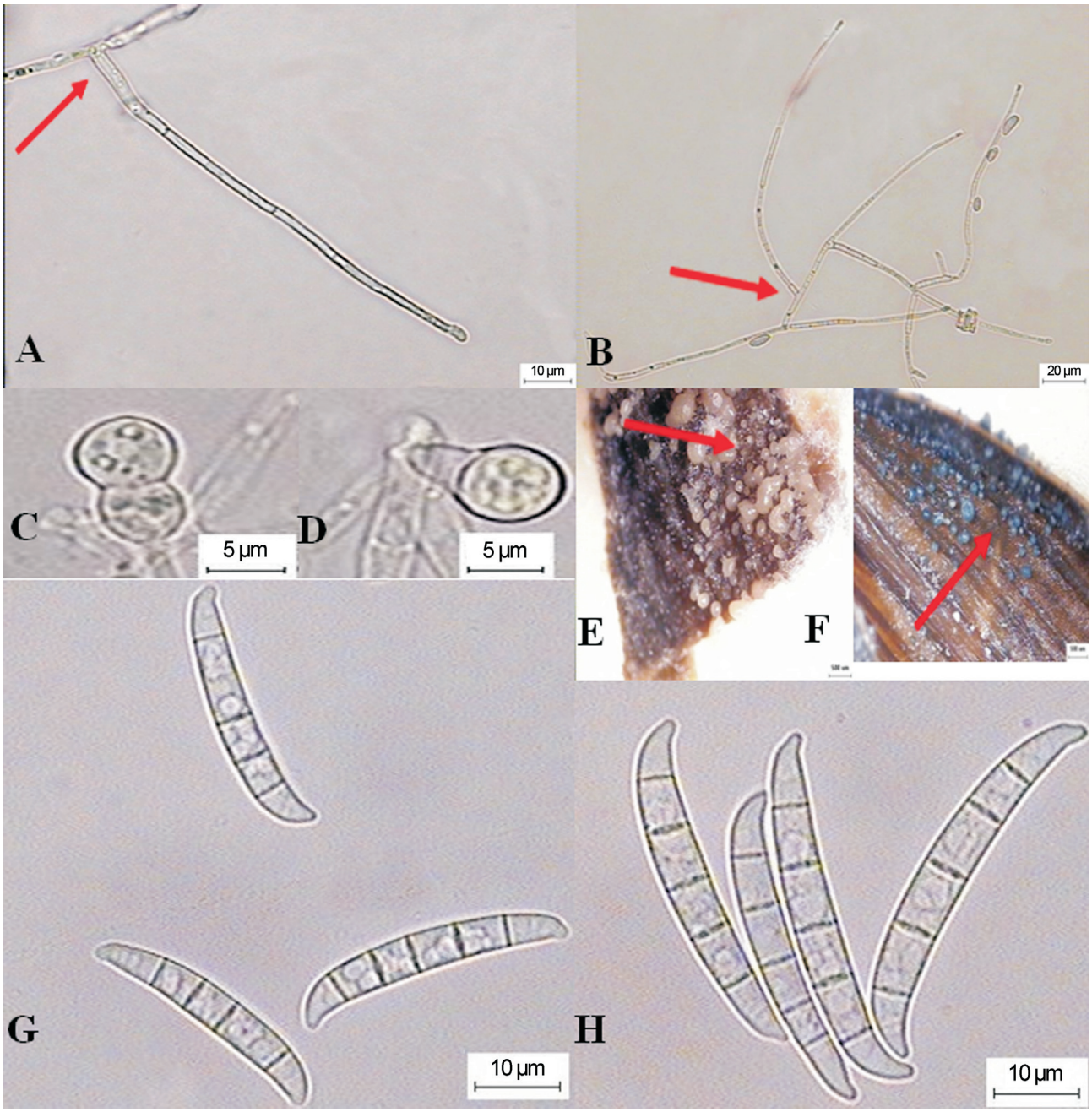

Figure 2 - A. Long monophialides commonly observed in *F. solani* morphotype II; B. Branched long monophialides observed only in *F. solani* morphotype I; C-D. Typical chlamydospores observed in both morphotypes I and II; E. Cream sporodochia produced by both morphotypes I and II; F. Blue sporodochia observed only in morphotype II; G. Macroconidia produced by morphotype I; H. Macroconidia produced by morphotype II.

II were consistent with the description by Booth (1971). Therefore, in the present study, isolates of *F. oxysporum* were clearly identified based on morphological characteristics.

IGS-RFLP analysis of *F. oxysporum*

The IGS region of 40 isolates of *F. oxysporum* was successfully amplified and the size was approximately 2500 bp. The IGS fragments generated were digestible by all seven restriction enzymes, except for *Pst*I where only three isolates of *F. oxysporum* (C9715L, J9807L, and J9806L) were digested. A total of 33 haplotypes were assigned among the 40 isolates comprising 1 to 4 isolates in each haplotypes.

The UPGMA cluster analysis clearly divided the isolates of *F. oxysporum* into two main clusters, A and B with percentage of similarity from 89% to 100%. The clusters were in accordance with Morphotypes I and II in which cluster A comprises 17 isolates of Morphotype I and cluster B consists of 23 isolates of Morphotype II. Both clusters

Table 2 - Macroscopic- and microscopic characteristics of morphotypes I and II of *F. oxysporum* isolates associated with CD of oil palm in Malaysia and Indonesia

Morphological characterization	*F. oxysporum*	
	Morphotype I	Morphotype II
Macroscopic characteristic		
Colony colour[a]	Pale, dark to peach- violet	White to white-violet
Pigmentation[b]	Pale, dark to peach-violet	Pale violet to colourless
Growth rate (cm)[c]	4.3 ± 0.7	4.5 ± 0.5
Microscopic characteristic		
Macroconidia morphology	Straight and relatively slender	Slightly curved and thick
Apical cell morphology	Tapered and curved	Tapered with slight hook
Basal morphology	Pointed	Foot shaped
Mean length of macroconidia (μm)[d]	42.2 ± 4.5	34.9 ± 2.1
Mean width of macroconidia (μm)[d]	3.9 ± 0.4	4.2 ± 0.3
Macroconidia septation	3-7 (3-septate most common)	3-5 (3-septate most common)

[a]Colony colour were determined by observing the upper surface of the colony.
[b]Pigmentation were determined by observing the lower surface of the colony.
[c]Growth rates were taken after 3 days of incubation at 25 °C.
[d]Mean values of length and width of 50 randomly picked macroconidia ± standard deviation.

have the percentage of similarity from 89% to 100% and 90% to 100%, respectively (Figure 5).

Similarly with *F. solani*, the occurrence of 33 IGS haplotypes indicated that the *F. oxysporum* isolates also were highly variable. For *F. oxysporum*, transposable elements could act as the generators of diversity in the fungal genome because their activity yields spontaneous mutations and consequently variability in the genome (McDonald, 1993; Daboussi and Langin, 1994). Edel *et al.* (2001) also reported that transposable elements in *F. oxysporum*, namely *impala* and *Fot*1 could be responsible for its diversity.

Two main clusters were obtained from UPGMA cluster analysis, which corresponded with the two Morphotypes of *F. oxysporum* and there was no correlation between the grouping of the isolates and the locations. The highly variable data from IGS-RFLP analysis of the *F. oxysporum* isolates also indicated that the isolates were highly variable and many workers have shown that *F. oxysporum* is a species complex (Kistler and Momol, 1990). Furthermore, phylogenetic analysis has shown that *F. oxysporum* comprised a number of distinct lineages (Fourie *et al.*, 2009). Other studies that used various types of DNA-based methods and vegetative compatibility group method also have reported high levels of genetic diversity within *F. oxysporum* isolates (Kim *et al.*, 2001; Groenewald *et al.*, 2006). IGS-RFLP analysis was shown to be effective for detecting genetic differences at intraspecific level (Appel and Gordon 1995) and has also shown to be a suitable method for interstrain relationship analysis within *F. oxysporum* and the genetic characterization of large populations of *F. oxysporum* (Edel *et al.*, 1997).

Burgess *et al.* (1994) stated that the observable differences of macroconidial sizes could exist depending on geographical region with different climatic conditions. In the present study, microclimate factors might contribute to the differences and the diversity in morphological characteristics observed and the results suggested that there is an extreme genetic diversity within the species (Kistler and Momol, 1990).

The high level of genetic diversity showed from the cultural and morphological characteristics of both *F. solani* and *F. oxysporum* isolates were in accordance with the highly variable IGS-RFLP analysis. Variation in the IGS regions could be due to the point mutation occurred at the recognition sites (Mishra *et al.*, 2002). Highly variable haplotypes may also indicate considerable divergence in IGS region which often related to both length and sequence variation (Hillis and Dixon, 1991). Other factors could be recombination phenomenon occurring on one or more chromosomes (Boehm *et al.*, 1994) and translocation process that happened during mitotic divisions (Appel and Gordon 1995). The occurrence of the two Morphotypes and highly variable IGS-RFLP analysis could also indicate that the isolates of *F. solani* and *F. oxysporum* associated with CD of oil palm in Malaysia and Indonesia comprised different subspecies or species as both taxa is regarded to represents species complexes (O'Donnell *et al.*, 2008).

Information on the genetic variability of *F. solani* and *F. oxysporum* could be used to study genetic population for disease-control management and breeding programs (Taylor *et al.*, 1999). The pathogen of CD of oil palm has not yet determined, although the initial assumption of the presence of both *F. solani* and *F. oxysporum* in the oil palm with CD

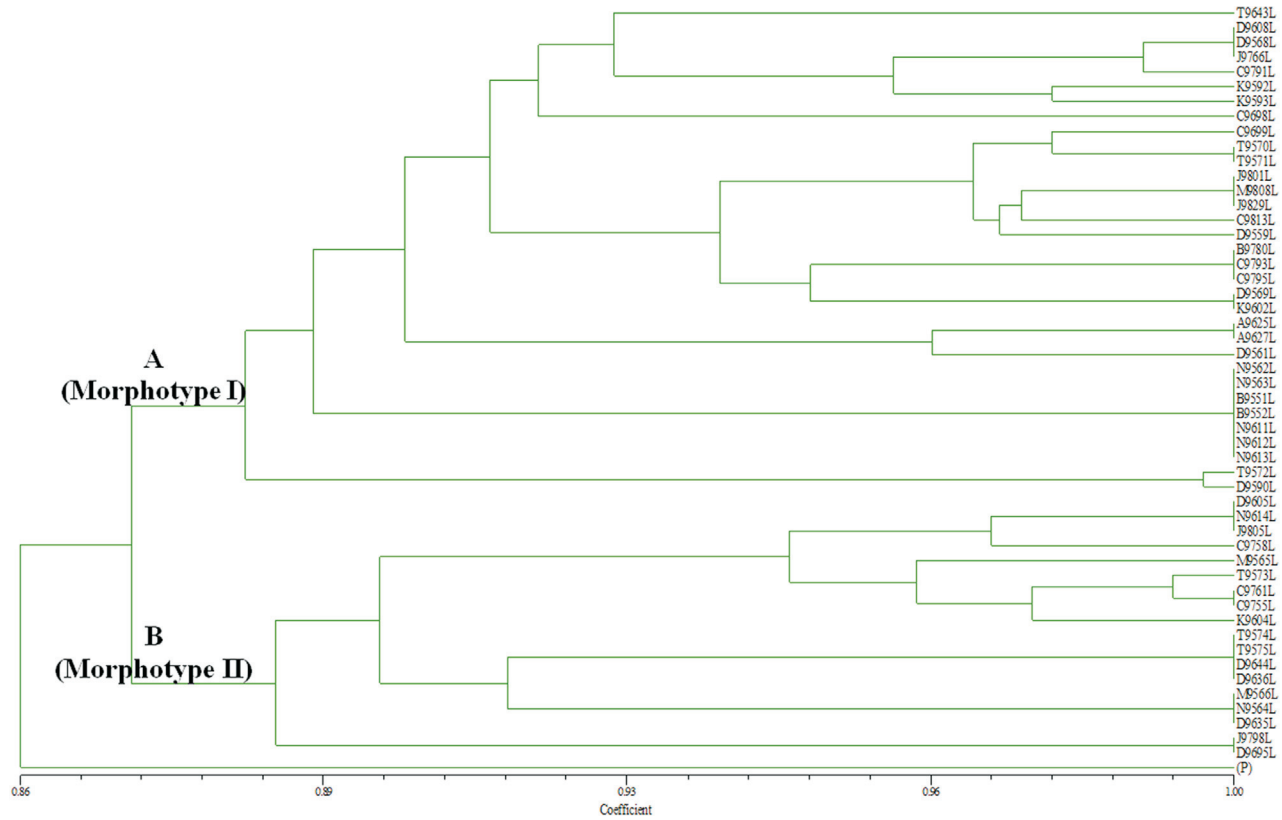

Figure 3 - UPGMA dendogram obtained by RFLP-IGS analysis of *F. solani* isolates associated with crown disease of oil palm in Malaysia. Two distinct clusters separating Morphotype I (cluster a) and Morphotype II (cluster b). *F. proliferatum* was used as an outgroup.

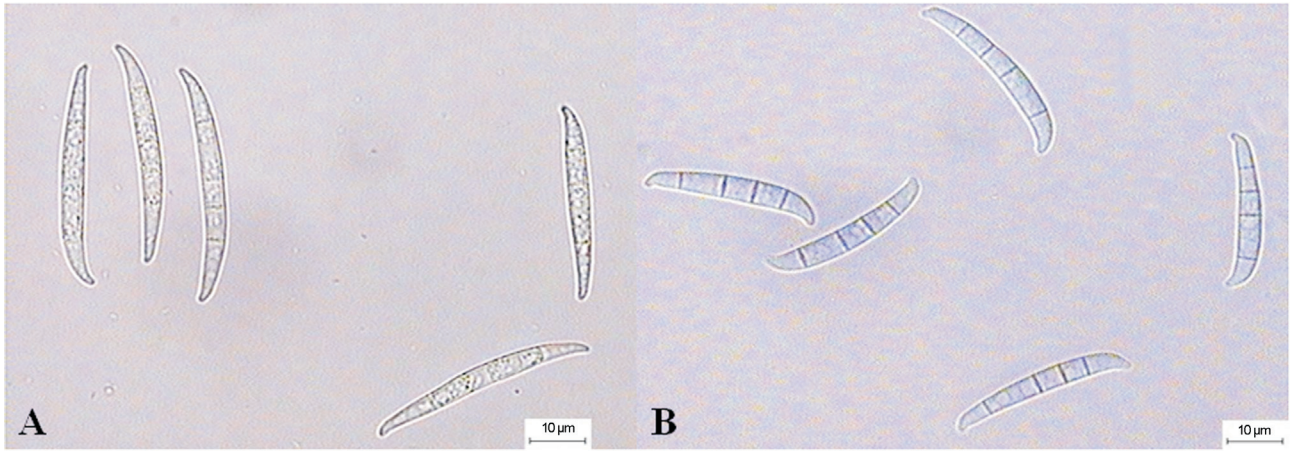

Figure 4 - A. Long and narrow macroconidia in isolates of morphotype I; B. Short and wide macroconidia in isolates of Morphotype II.

symptoms might be as endophytes. Therefore once pathogenicity test is conducted and if *F. solani* and/or *F. oxysporum* is proven to be the pathogen causing CD of oil palm, isolates from a particular forma specialis can be further subdivided according to their variation in virulence by assigning pathotypes to pathogenic races (Armstrong and Armstrong, 1981).

Fungal populations with high levels of genetic variations are generally more likely to adapt rapidly to different environmental conditions (McDonald and McDermott, 1993) and it has been reported that the appearance and progress of CD in oil palm is prone to the changes in the environmental factors and certain agronomic practices such as poor soil aeration, poor drainage, and unbalanced nutrition such as potassium shortage (Chinchilla *et al.*, 1997).

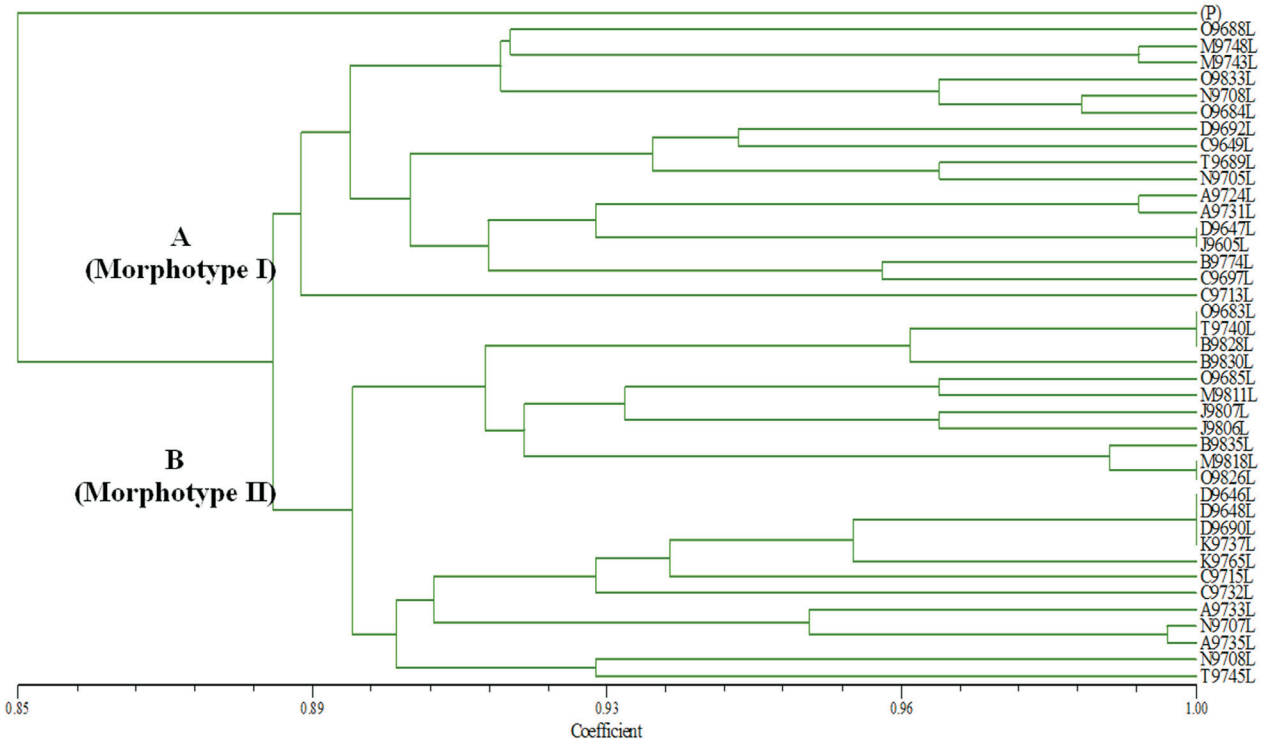

Figure 5 - UPGMA dendogram obtained by RFLP-IGS analysis of *F. oxysporum* isolates associated with crown disease of oil palm in Malaysia and Indonesia. Two distinct clusters separating Morphotype I (cluster a) and Morphotype II (cluster b). *F. proliferatum* was used as an outgroup.

This situation could promote *F. solani* and *F. oxysporum* to adapt with the environment they need to survive, and at this point, changes in genetic structure could lead to genetic diversity that might caused the previously unknown pathogenic ability of both *F. solani* and *F. oxysporum* on CD, become pathogenic, and eventually a new and serious disease of oil palm. Gordon and Okamoto (1992) speculated that those pathogenic isolates of *Fusarium* may arise from endophytic or non-pathogenic isolates.

The present study contributed to the knowledge on the diversity of *F. solani* and *F. oxysporum* associated with CD of oil palm. However, molecular phylogeny using Genealogical Concordance Phylogenetic Species Recognition (GCPSR) as suggested by Taylor *et al.* (2000) could be used to properly define both *F. solani* and *F. oxysporum* in a taxonomic or systematic sense. Further analysis involving *F. solani* and *F. oxysporum* isolates from Malaysia and other isolates from other oil palm growing regions such as Africa and South America would be beneficial in order to properly characterize *F. solani* and *F. oxysporum* as each one of them is a species complex that might comprises a new subspecies or species although the length and width of macroconidia appears to be convincing characters in species differentiation (Booth, 1971). In addition, the role of both *F. solani* and *F. oxysporum* associated with the CD symptoms in oil palm should also be clarified eventhough it is for now assumed that CD is a genetic disorder.

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